THE VITELLINE COAT OF THE MYTILUS EGG. I. NORMAL STRUCTURE AND EFFECT OF ACROSOMAL LYSIN

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The first study of a lysin extractable from *Mytilus* spermatozoa was made by Berg (1950), who distinguished two types of lytic activity: a "membrane-dissolving" action, which was described as attacking an outer layer of the egg surface made microscopically visible by plasmolyzing the eggs, and a "cement-dissolving" activity, which causes the first two blastomeres to become partially or completely separated.

Wada, Collier and Dan (1956) showed that the lysin in question is a component of the intact acrosome which is released into the medium when the acrosome is induced to react.

Colwin and Colwin (1960a, b) have also investigated the effect of a lysin extracted from the spermatozoa of *Hydroides hexagonus* on the egg of this species. Using thin sections and electron microscopy, they found that the lysin dissolves the middle, and major, component of the thick vitelline coat investing the cytoplasmic surface, although it appears not to affect the outer and inner borders of this envelope.

A report concerning the structure of the *Mytilus edulis* "egg membrane" has recently been published by Mancuso (1960). This author used a fixing solution which included formalin, acetone, acetic acid and sometimes chromic acid, as well as osmium tetroxide; the images observed in the electron microscope after this fixation led him to certain conclusions which differ considerably from those reached in this study. None of these differences, however, is so radical that it cannot be attributed to the effect of the fixative.

The present investigation was undertaken to observe the fine structure of the *Mytilus* egg surface and determine in detail how the acrosomal lysin affects it after fertilization, particularly in connection with the role of the vitelline coat in controlling the pattern of the first cleavage, and the shape and mutual relations of the first two blastomeres.

Material and Methods

Mytilus edulis from the Tokyo area is readily induced to spawn by keeping freshly collected animals dry in a refrigerator for several hours and then placing them in sea water at room temperature (20–23°), or by raising the temperature of the running sea water about 5° (to 18–20°) and administering an electrical stimulus, according to the method of Iwata (1949). Stimulated animals are returned to sea water in separate containers, and males which have begun to shed are stood, broad end downward, in a dry beaker to obtain concentrated sperm suspensions.

Pooled sperm from several males was used as the source of the acrosomal lysin. If 1 ml. of 0.36 *M* $CaCl_2$ is added to 9 ml. of rather concentrated sperm suspension, most of the spermatozoa undergo a reaction of the acrosome (see Wada *et al.*, 1956).

The sperm cells were removed by 10 minutes' centrifugation at 12,000 g (0°), and the clear supernatant was dialyzed against running sea water and used as the lysin. This solution retains its lytic activity indefinitely if it is kept frozen.

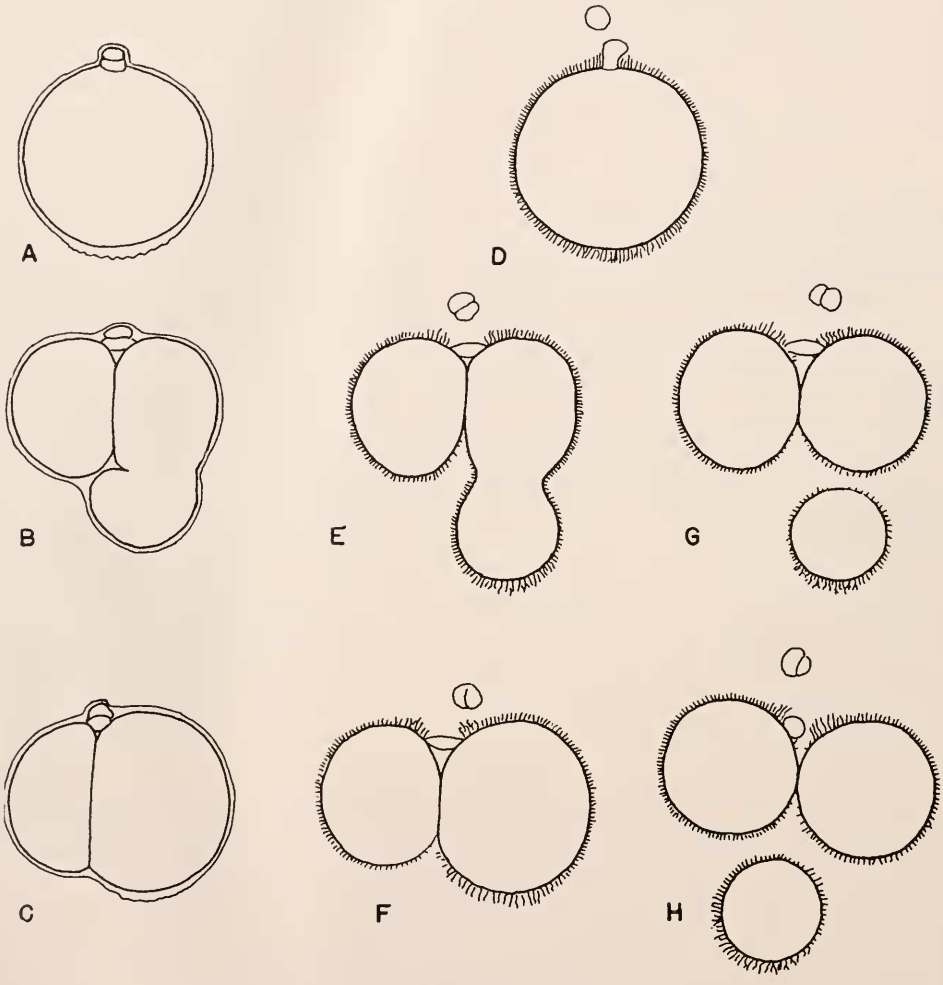


FIGURE 1. Camera lucida drawings of *Mytilus edulis* eggs cleaving under various conditions. A–C: in sea water; D–F: suspended in strong solution of acrosomal lysin; G, H: membrane removed by lysin and eggs transferred to calcium-free sea water. A, D: shortly before cleavage; B, E, G: mid-cleavage trefoil stage; C, F, H: interphase between first and second cleavages. Microvilli are visible with phase contrast as striated "halo."

All fixation was done at room temperature with 1% OsO_4 in sea water. The egg suspensions were fixed for 30 minutes, washed and post-fixed in 5% formalin-sea water for several hours, embedded in methacrylate, sectioned with a Porter-Blum microtome and observed with a JEM-5G electron microscope.

Results

Living eggs

The unfertilized *Mytilus* egg is irregularly oval, and is surrounded by a conspicuous hyaline zone about 1 μ thick, which is referred to as the vitelline membrane in Field's original description (1921–1922). Outside of this "membrane" is a rather thin (7–10 μ) layer of transparent material ("jelly") which can most easily be detected by adding india ink to the egg suspension (see Wada *et al.*, 1956; Fig. 6).

On being fertilized, the egg immediately becomes spherical (diameter about 63 μ), but no change can be observed in the surface layers except that by the time of the first cleavage, the thickness of the vitelline coat appears to increase slightly.

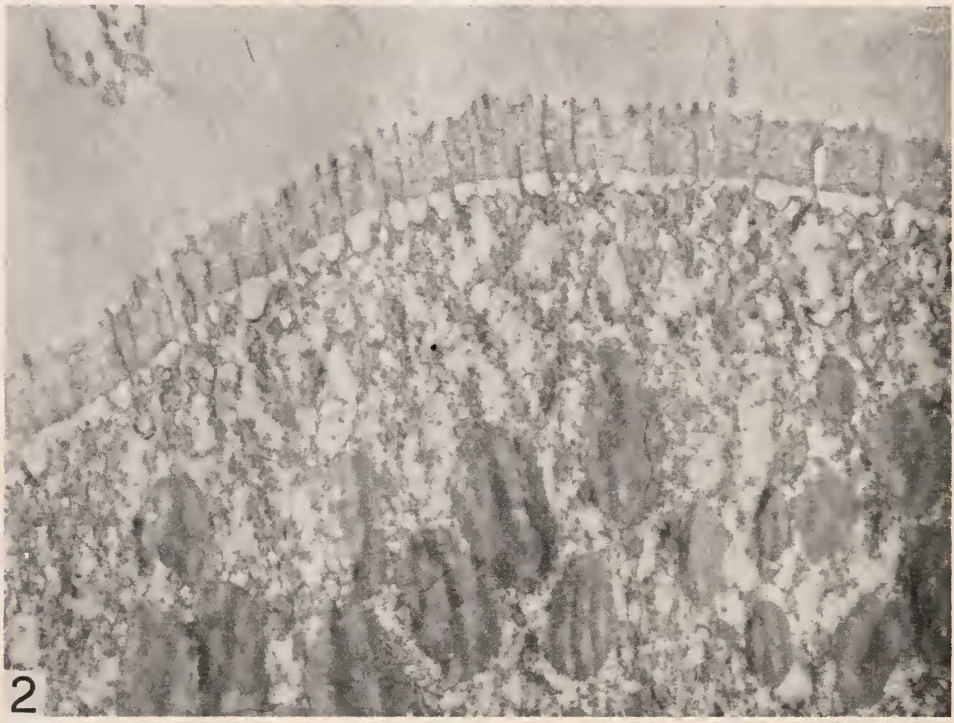
When the polar bodies are extruded, they lie under the vitelline coat, flattened against the cytoplasmic surface (Fig. 1A). At the first cleavage the egg forms a polar lobe; as cleavage proceeds, this lobe is compressed against the opposite, AB, blastomere by the tension exerted by the vitelline coat (Fig. 1B). Once cleavage is complete, the polar lobe material flows back into the CD cell, and the two blastomeres become closely apposed during the succeeding interphase (Fig. 1C).

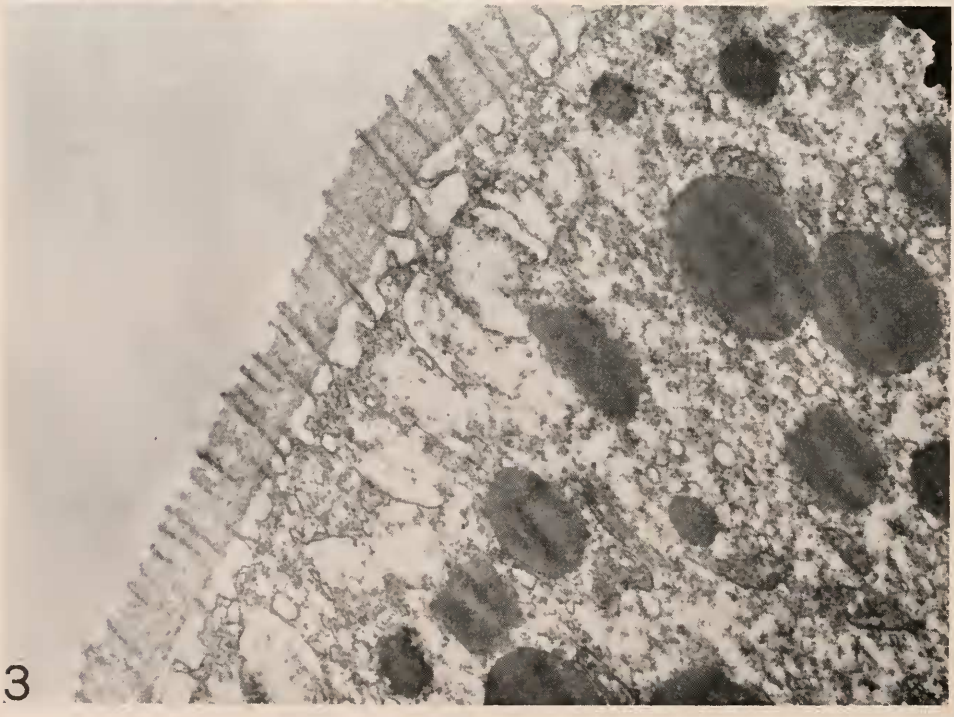
If an egg is treated with acrosomal lysin 10 minutes after fertilization and continuously observed with phase contrast, it is seen that the vitelline coat loses first its sharp outline and then its hyaline refringency, and finally gives place to a layer of fine processes which cover the whole surface of the cell. These processes are clearly longer at the vegetal side of the egg, and also in a restricted area at the animal pole (Fig. 1D).

The first polar body bulges out freely as it is formed, and drifts away from the egg if the preparation is jarred. The second polar body remains attached to the egg surface, the first polar body usually dividing as the second is formed. At cleavage, the polar lobe extends out at right angles to the mitotic spindle (Fig. 1E); the connection between polar lobe and CD blastomere is narrower than normal, and in very strong lysin or when the eggs are transferred to calcium-free sea water after strong lysin, the connection is often severed (Fig. 1G). As Berg has reported (1950), the two resting blastomeres tend to be more spherical than those of the controls, especially after extended exposure to strong lysin, although a considerable degree of contact is more common than complete separation (Fig. 1F, H) (see also Berg, 1950; Pl. 1, c, d; Wada *et al.*, Fig. 7).

Electron microscopy

Normal egg surface. Thin sections of the unfertilized *Mytilus* egg (Fig. 2) show that its surface is similar to that of the egg of another bivalve mollusc, *Spisula*, according to an electron micrograph by Rebhun (Allen, 1958). The cytoplasmic surface is extended into fine microvilli of a relatively uniform size and regular distribution, 0.7–1 μ in length, and usually straight, although two may be connected at their bases to give an effect of branching. These microvilli extend into and through a rather dense layer, about 0.5 μ thick, of homogeneous material of the sort described by the Colwins as "felt-like," which is obviously the hyaline component of the vitelline coat as observed with light microscopy. The tips of the microvilli protrude slightly beyond the outer surface of this layer; its conspicuously smooth inner surface is separated by a definite perivitelline space from the outer border of





FIGURES 2–3.

the cytoplasmic mass between the bases of the microvilli. From the tips of the microvilli numerous extremely fine fibrils extend outward, constituting at least one component of the so-called jelly layer.

As can be seen in Figures 2, 3 and 8, no formation which could be described as a "membrane" lies outside this layer of hyaline material, although the micrographs show regions of greater absorption, as at the right of Figure 2, which suggest that the surface of the vitelline coat is somewhat denser than its interior. A similar but more pronounced condensation is apparent in Rebhun's micrograph of the *Spisula* egg, and Mancuso's figures of the *Mytilus* egg show most of the hyaline substances concentrated into two layers, corresponding to the inner and outer surfaces of what appears in this study as a nearly homogeneous matrix.

The cytoplasm is bounded by a plasma membrane, which is continuous with the walls of the microvilli. Beneath this is a region of cortical cytoplasm 1–1.5 μ thick, generally free from yolk granules but containing conspicuous spherical membranes which in Mancuso's micrographs includes a substance having an electron absorbancy somewhat greater than that of the yolk.

Fertilization induces no changes in any of these structures that can be detected in the electron micrographs (Fig. 3). In eggs fixed between 20 and 70 minutes after fertilization, however, it is found that on parts of the egg surface, the bases of adjacent microvilli have united, while the wider spaces between these villous trunks have also deepened. The microvilli thus come to present an overall appearance of branching, but careful observation shows that their dimensions and arrangement within the vitelline coat are the same as those of the unfertilized eggs; what has changed is the intervillous cytoplasmic surface. This new state of affairs can be observed in the untreated living egg as a thickening of the vitelline coat, as mentioned above, and the greater length in the polar regions of the processes observed after lysis of the hyaline layer material is due to an extreme expression of this tendency, in all probability connected with the special role of these areas in polar lobe and polar body formation.

Lysin-treated eggs. Sections of eggs fixed after having been exposed to lysin for one minute (Fig. 4) show that the hyaline material of the vitelline coat has been evenly attacked by the lysin—*i.e.*, dissolution of the material has taken place rather uniformly throughout the layer. After an exposure of two minutes (Fig. 5), the material is virtually all dissolved, except for some vague remnants of it left clinging to the microvilli; the latter remain exposed as straight, unbranched processes, continuous with the main body of the cytoplasm and apparently unaffected by the lysin. The fine fibrils of the jelly layer are also intact (*cf.* Wada *et al.*, 1956; Fig. 7), and with the hyaline substance removed, it can be seen that there are short fibrils of the same kind projecting from the sides of the microvilli.

When eggs are exposed to the lytic solution for 10 minutes (Fig. 6), the hyaline material is completely dissolved, whereas the microvilli and the fine fibrils of the jelly are quite unaffected. The portion of the egg surface appearing in Figure 6 is

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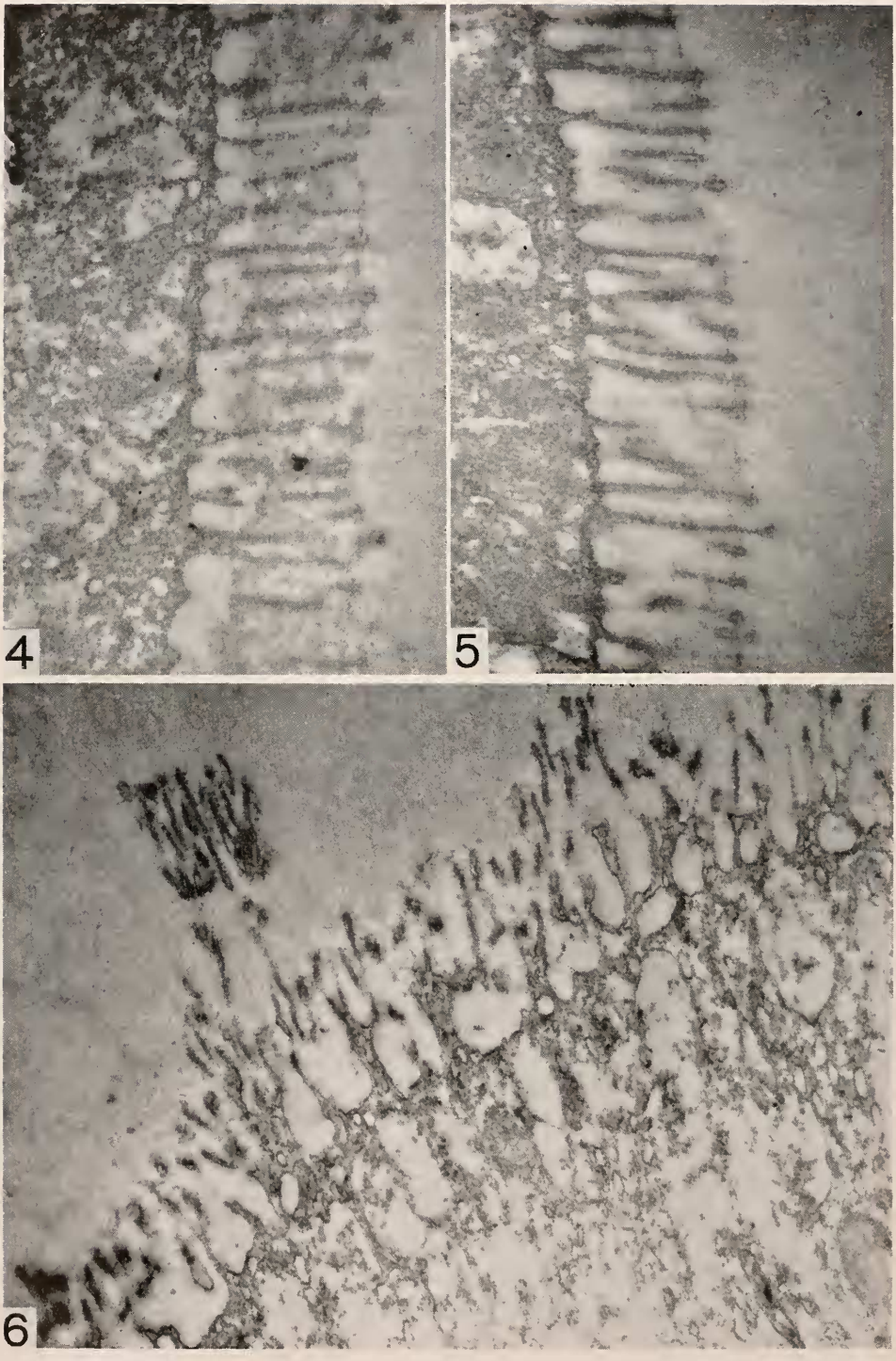
FIGURE 2. Surface of unfertilized *Mytitus* egg, showing vitelline coat consisting of hyaline material supported by microvilli. × 16,000.

FIGURE 3. Surface of fertilized *Mytitus* egg fixed 10 minutes after insemination. Note fine fibrils of "jelly layer" and empty membranes of cortical granules which have been extracted during preparation (see text). × 16,000.



FIGURES 4–6.

apparently from the vegetal region, since it represents an extreme case of the "branching" effect.

Further exposure to the lytic activity, up to 60 minutes, still leaves the microvilli and their fibrils unaffected (Fig. 7). The cytoplasmic protuberances carrying the microvilli in this section are coarser than those shown in Figure 6; it is not clear whether this represents a topographical characteristic or is the result of exposing the cytoplasmic surface without its supporting coat for a long period.

Lysin plus calcium-free sea water. To investigate the effect of lack of calcium on these surface structures, fertilized eggs were transferred to calcium-free artificial sea water [1] 10 minutes after insemination, as controls for another lot of fertilized eggs which were first exposed to lysin for 10 minutes and then washed with calcium-free sea water and left in it for 50 minutes (fixation at 70 minutes after insemination, shortly before beginning of first cleavage).

The calcium-free controls (Fig. 8) show no differences from the sea water controls, indicating that the integrity of the vitelline coat in these eggs is not dependent on the presence of calcium in the medium. The fibrils of the jelly layer are also found intact, both in the controls and in the sample of eggs exposed to lysin followed by calcium-free sea water (Fig. 9), and no special effect of the lack of calcium on the denuded cytoplasmic surface can be observed.

Discussion

The general structure of the *Mytilus* egg surface, as seen at high magnification, bears a surprisingly close resemblance to the surface complex of the fertilized sea urchin egg, in which secondarily-formed microvilli extend into and attach the egg surface to a hyaline layer similarly consisting of a homogeneous material (Endo, 1961). In both these systems, the overlying layer of the hyaline substance supports the cytoplasmic surface and controls the shape of the embryo as it develops through the cleavage stages.

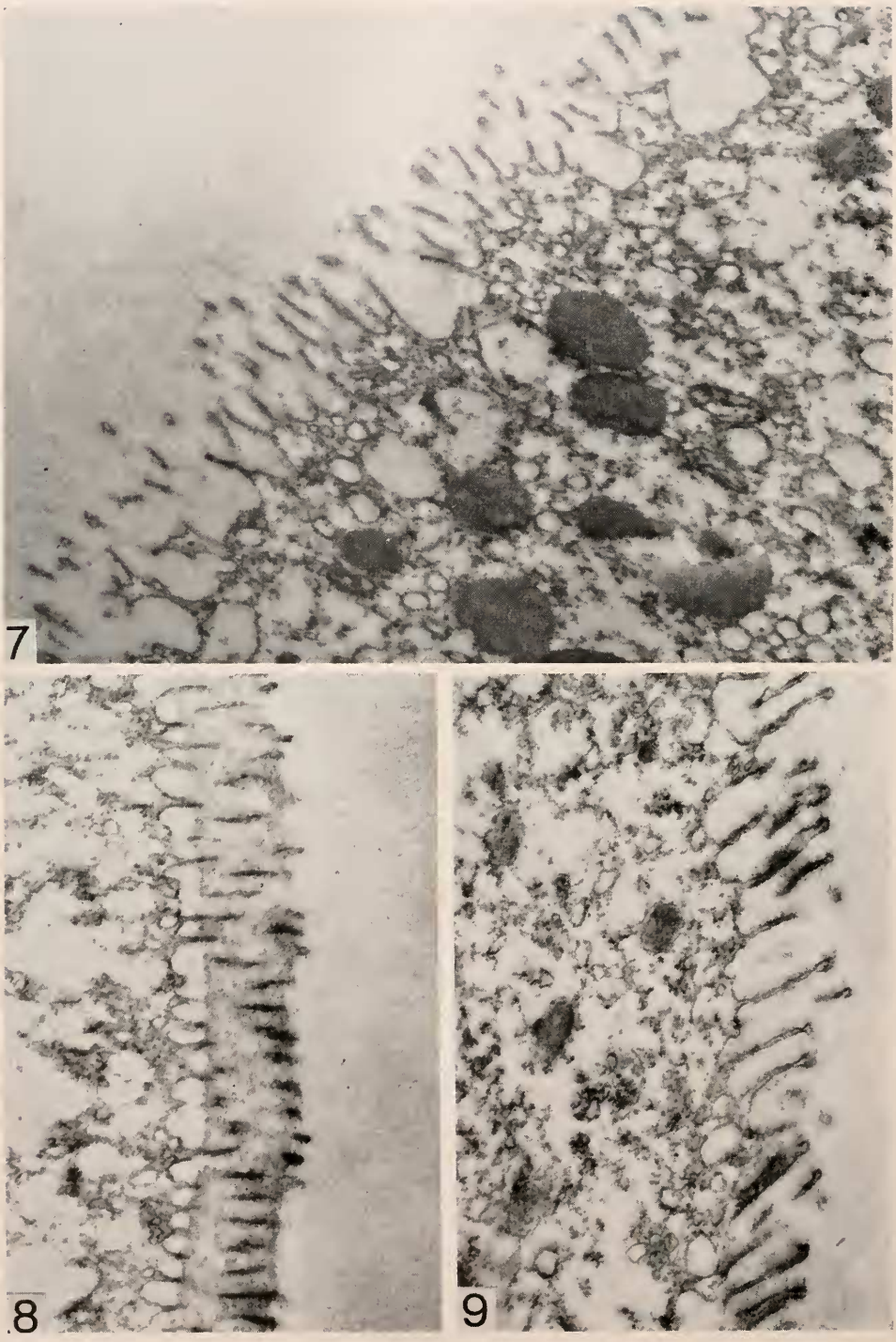
Experiments of the sort first performed by A. R. Moore (1940), showing that sucrose freely penetrates the hyaline layer of echinoderm embryos during the cleavage stages, furnish evidence that the osmotic properties of this layer are less exclusive than those of cytoplasmic membranes. On the other hand, the fact that the width of the echinoderm hyaline layer is observed to increase just before each of the early cleavages (Dan, 1952) indicates that some osmotically active substances are retained within it. The observation presented in this study, that the *Mytilus* egg surface proper becomes indented so that the microvilli come to project from the summits of thicker cytoplasmic protuberances, suggests that a similar osmotic process is at work in these eggs, causing an increase in the volume of the perivitelline fluid. In view of the extent to which the dividing egg departs from the spherical shape, especially as it forms and retracts the polar lobes, the intervention of some such

Figure 4. Surface of fertilized *Mytilus* egg fixed after exposure of one minute to strong acrosomal lysin. Hyaline material partly dissolved. × 24,000.

Figure 5. Hyaline material almost completely dissolved after two-minute exposure to lysin. Note that microvilli and fibrils of jelly layer are unaffected. × 24,000.

Figure 6. Vegetal surface of fertilized *Mytilus* egg after 10-minute exposure to lysin. Hyaline material completely removed; microvilli and fibrils unaffected. × 16,000.

[1] Dan's (1954) "chloride mixture No. I."



FIGURES 7–9.

device to reduce the restraining effect of the vitelline coat on the cytoplasmic surface would seem to be an essential prerequisite for cleavage.

That the tensile properties of the intact *Mytilus* egg surface are chiefly due to the hyaline material of its vitelline coat is suggested by the separation of the polar bodies and the considerable change in the configuration of the first cleavage following lysis of this layer (in Figure 1, compare D and E with A and B). On the other hand, the vitelline coat must be capable not only of expanding to some extent, but also of being contracted to a comparable extent, since the formation of the polar lobes involves an increase in surface area, while their retraction causes it to decrease. When the eggs are not in the best condition, wrinkling of the vitelline coat, or its complete separation from the plasma membrane as a large blister at the vegetal pole, attests to the failure of such contraction.

It is clear that the activity of the lysin derived from the sperm acrosome is specifically directed against the hyaline material of the vitelline coat, and has no effect, even after 60 minutes, on the plasma membrane. Comparing Figures 6 and 7, which both show areas of the vegetal surface, it at first appears as though prolonged exposure to the lysin has weakened the egg surface so that the slender processes supporting the clusters of microvilli in Figure 6 spread out into the thick, poorly organized protuberances seen in Figure 7. It is necessary to consider, however, that during this period the polar lobes associated with first and second polar body formation have caused the expansion of this surface in the absence of the vitelline coat, which would normally have held the distal parts of the microvilli in a fixed arrangement. It therefore seems probable, especially since the microvilli remain unchanged even after prolonged exposure, that the observed effect is secondarily produced by the absence of the supporting layer, rather than primarily, by some action of the lysin on the cytoplasmic surface.

The long microfibrils which arise from the tips of the microvilli and constitute what has been thought of as the jelly layer are interesting because of their resistance to the dissolving actions of lysin and of calcium-free sea water, and because they are fixed by osmium. The two latter characteristics set them in contrast to the mucopolysaccharide jelly of the sea urchin egg, and suggest that the zone around the *Mytilus* egg consisting of these massed fibrils should not be thought of in the same terms unless evidence can be found to indicate the presence of a more labile component.

The result of the present investigation supports the doubt which was expressed in the earlier study (Wada *et al.*, 1956) concerning Berg's (1950) suggestion that the AB and CD blastomeres are held together by a cementing substance, presumably secreted in the furrow region of the cleaving egg. It seems evident that it is rather the restraint exerted by the encircling vitelline coat which presses the blastomeres against each other in normal cleavage. That this is not the whole explanation,

FIGURE 7. Surface of *Mytilus* egg after 60-minute exposure to strong acrosomal lysin. Microvilli and fibrils still unaffected; structure of cytoplasmic surface somewhat modified as result of prolonged absence of supporting layer of hyaline material. × 16,000.

FIGURE 8. Vitelline coat of fertilized *Mytilus* egg transferred to calcium-free sea water 10 minutes after insemination; fixed at 70 minutes. Note that hyaline material and fibrils of jelly layer are both resistant to lack of calcium. × 16,000.

FIGURE 9. Surface of fertilized *Mytilus* egg exposed for 10 minutes to strong lysin, washed in calcium-free sea water and left in this medium until just before first cleavage; fixed 70 minutes. × 16,000.

however, is shown by the observation that even when the hyaline component of this layer has been dissolved, the blastomeres preserve a considerable degree of mutual contact (Fig. 1F) unless cleavage takes place in calcium-free sea water (Fig. 1G, H).

If an analogy may be drawn between these cells and sea urchin blastomeres, which also normally have their outer surfaces attached by cytoplasmic processes to a hyaline layer (Dan and Ono, 1952), the extreme sphericity of the *Mytilus* blastomeres in calcium-free sea water after removal of their vitelline coat can be explained as an abnormal equalization of the post-cleavage membrane tension involving the whole weakened (by the absence of calcium) surfaces of the blastomeres, instead of the usual localization of such stretching in the furrow region (Dan, 1954).

Since electron microscopy shows that the outermost covering of the *Mytilus* egg is a single layer of what can be called a cementing substance or matrix material supporting and fixing in regular arrangement a brush of microvilli, rather than any structure which conforms with the usual concept of "membrane," it appears that the two lytic activities suggested by Berg (membrane-lytic and cement-lytic) would be better described as degrees of effectiveness of a single activity, combined with secondary effects of variable experimental conditions such as the degree of calcium deficiency and the length of the period during which the cytoplasmic surface is without its supporting layer.

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Summary

1. Electron microscopy shows the egg of *Mytilus edulis* to be surrounded by a vitelline coat consisting of a layer about 0.5 μ thick, which corresponds to the refringent hyaline zone seen with the light microscope. This layer has a smooth inner surface, separated from the cytoplasmic surface proper by a space about 0.2 μ wide. The plasma membrane forms a brush of regularly arranged, straight microvilli 0.7–1 μ in length. These pass through and protrude slightly beyond the outer surface of the hyaline material, where their tips give rise to numerous extremely delicate fibrils which constitute at least one component of the "jelly layer." Fertilization does not cause any visible changes in these structures of the egg surface.

2. Exposure of a fertilized egg for one minute to a strong solution of acrosomal lysin causes an evident dissolution of the hyaline substance, and a two-minute exposure removes it almost completely, leaving the microvilli exposed but otherwise unaffected. The fibrils of the jelly layer also resist the lytic action. Exposure to lysin for 60 minutes induces no further changes in these structures.

3. It is concluded that the acrosomal lysin is specific for the single substance constituting the hyaline portion of the vitelline coat, and that the layer composed of this material is chiefly responsible for the configuration of the cleaving egg and the close contact of the blastomeres after cleavage.

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